

Contents lists available at [ScienceDirect](http://ScienceDirect.com)International Journal for Parasitology:
Drugs and Drug Resistancejournal homepage: www.elsevier.com/locate/ijpddrProfiling the anti-protozoal activity of anti-cancer HDAC inhibitors
against *Plasmodium* and *Trypanosoma* parasitesJessica A. Engel^a, Amy J. Jones^a, Vicky M. Avery^a, Subathdrage D.M. Sumanadasa^a,
Susanna S. Ng^a, David P. Fairlie^b, Tina S. Adams^a, Katherine T. Andrews^{a,*}^a Eskitis Institute for Drug Discovery, Griffith University, Queensland, Australia^b Institute for Molecular Bioscience, The University of Queensland, Brisbane, Q4072, Australia

ARTICLE INFO

Article history:

Received 18 February 2015

Received in revised form

8 April 2015

Accepted 12 May 2015

Available online 20 June 2015

Keywords:

*Plasmodium falciparum**Trypanosoma brucei*

Malaria

African sleeping sickness

Histone

HDAC inhibitors

ABSTRACT

Histone deacetylase (HDAC) enzymes work together with histone acetyltransferases (HATs) to reversibly acetylate both histone and non-histone proteins. As a result, these enzymes are involved in regulating chromatin structure and gene expression as well as other important cellular processes. HDACs are validated drug targets for some types of cancer, with four HDAC inhibitors clinically approved. However, they are also showing promise as novel drug targets for other indications, including malaria and other parasitic diseases. In this study the *in vitro* activity of four anti-cancer HDAC inhibitors was examined against parasites that cause malaria and trypanosomiasis. Three of these inhibitors, suberoylanilide hydroxamic acid (SAHA; vorinostat[®]), romidepsin (Istodax[®]) and belinostat (Beleodaq[®]), are clinically approved for the treatment of T-cell lymphoma, while the fourth, panobinostat, has recently been approved for combination therapy use in certain patients with multiple myeloma. All HDAC inhibitors were found to inhibit the growth of asexual-stage *Plasmodium falciparum* malaria parasites in the nanomolar range (IC₅₀ 10–200 nM), while only romidepsin was active at sub- μ M concentrations against bloodstream form *Trypanosoma brucei brucei* parasites (IC₅₀ 35 nM). The compounds were found to have some selectivity for malaria parasites compared with mammalian cells, but were not selective for trypanosome parasites versus mammalian cells. All compounds caused hyperacetylation of histone and non-histone proteins in *P. falciparum* asexual stage parasites and inhibited deacetylase activity in *P. falciparum* nuclear extracts in addition to recombinant PfHDAC1 activity. *P. falciparum* histone hyperacetylation data indicate that HDAC inhibitors may differentially affect the acetylation profiles of histone H3 and H4.

© 2015 The Authors. Published by Elsevier Ltd on behalf of Australian Society for Parasitology. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Tropical parasitic diseases result in > 1 million deaths annually (WHO, 2008). The lack of vaccines and safe and efficacious drugs that are not hampered by parasite drug resistance is driving the search for new therapies. Targeting regulatory processes essential for parasite growth and development, such as protein acetylation (Andrews et al., 2012a, 2012b; Kelly et al., 2012) and methylation (Malmquist et al., 2012; Sundriyal et al., 2014), represents a promising starting point for identifying new anti-parasitic drugs. This strategy has been used successfully for other diseases, such as certain types of cancer, where inhibitors that target lysine

deacetylase enzymes (histone deacetylase (HDAC) inhibitors) have been approved for clinical use (Grant et al., 2007; Campas-Moya, 2009; Thompson, 2014). HDAC inhibitors are also under investigation for parasitic diseases such as malaria, trypanosomiasis, schistosomiasis and leishmaniasis (Murray et al., 2001; Ingram and Horn, 2002; Mai et al., 2004; Sheader et al., 2004; Azzi et al., 2009; Dubois et al., 2009; Patil et al., 2010; Wang et al., 2010; Alonso and Serra, 2012; Andrews et al., 2012a; Kelly et al., 2012; Carrillo et al., 2015; Stolfi et al., 2014).

HDAC inhibitors target eukaryotic HDAC enzymes which work together with histone acetyltransferases (HATs) to reversibly modify the acetylation state of histone and non-histone proteins (Khan and La Thangue, 2012). As a consequence, these enzymes regulate chromatin structure and gene expression (De Ruijter et al., 2003) and other important cellular processes such as cell cycle regulation and apoptosis (Khan and La Thangue, 2012). In human

* Corresponding author. Eskitis Institute for Drug Discovery, Don Young Road, Building N.75, Griffith University, Nathan, 4111, Queensland, Australia.

E-mail address: k.andrews@griffith.edu.au (K.T. Andrews).

cells, 18 HDACs have been identified (reviewed in (Mariadason, 2008)) and classified according to their co-factor dependency on either zinc or NAD^+ and sequence homology to yeast proteins (reviewed in (Xu et al., 2007)). In *Plasmodium falciparum*, five HDAC encoding genes have been annotated (PlasmoDB gene IDs: PF3D7_0925700 (*Pfhdac1*), PF3D7_345 1472200 (*Pfhdac2*), PF3D7_1008000 (*Pfhdac3*; *Pfhda2* (Coleman et al., 2014)), PF3D7_1328800 (*Pfsir2a*), and PF3D7_1451400 (*Pfsir2b*) (Aurrecoechea et al., 2009)). Three of the proteins/predicted proteins encoded by these genes have homology to class I (*PfHDAC1*) and II (*PfHDAC2* and 3) mammalian HDACs, while the remaining two are class III HDACs, or silent information regulator 2 (SIR2) proteins. *PfSir2A* and B are not essential to the growth and development of asexual intraerythrocytic *P. falciparum* parasites (Freitas-Junior et al., 2005; Tonkin et al., 2009). While less is known about the class I/II HDAC homologues, knock-down of *PfHDAC3* (*PfHDA2*) inhibits asexual-stage growth and development (Coleman et al., 2014). In *T. b. brucei*, four putative class I/II HDAC orthologues (*TbDAC* 1–4) have been identified, with *TbDAC1* and 3 being essential in bloodstream forms of the parasite (Ingram and Horn, 2002). The crucial role these enzymes play in modulating the acetylation state of histone proteins and gene expression (Xu et al., 2007) makes them attractive targets for novel therapeutic intervention strategies.

Several experimental HDAC inhibitors have shown promising activity *in vitro* (50% inhibitory concentrations (IC_{50}) ~0.01–0.3 μM) and *in vivo* against *Plasmodium* parasites, with some showing high selectivity for the parasite versus mammalian cells (reviewed in (Andrews et al., 2012b)). In contrast, only limited literature is available in regards to HDAC inhibitor activity against trypanosomes, with all compounds examined to date appearing non-selective (Ingram and Horn, 2002; Sheader et al., 2004; Wang et al., 2010; Alonso and Serra, 2012; Kelly et al., 2012; Carrillo et al., 2015). In this study we adopted a “piggyback” approach (Nwaka and Hudson, 2006) to extend these findings, focussing on four HDAC inhibitors (Fig. 1) that are clinically approved for cancer. Vorinostat (SAHA; Sigma Aldrich, USA), romidepsin (FK228; Selleck Chemicals, USA), and belinostat (Beleodaq; Spectrum Pharmaceuticals, Inc., USA) have been approved for the clinical treatment of cutaneous or peripheral T-cell lymphoma (Grant et al., 2007; Prince and Dickinson, 2012; Thompson, 2014) and are undergoing clinical

trials for various other cancers, including prostate and epithelial ovarian cancers (Garcia-Manero et al., 2008; Modesitt et al., 2008; Molife et al., 2010; Coiffier et al., 2012). Vorinostat and belinostat are hydroxamate-based pan-inhibitors of class I and II mammalian HDACs (Grant et al., 2007; Steele et al., 2011), while romidepsin is a cyclic tetrapeptide pro-drug (Sandor et al., 2002; Byrd et al., 2005) and has some HDAC isoenzyme-selectivity for class I mammalian HDACs (Prince and Dickinson, 2012). Panobinostat (LBH-589; Selleck Chemicals, USA) is a hydroxamate-based pan-HDAC inhibitor that acts on class I, II and IV HDACs (Prince et al., 2009). Panobinostat has recently been approved for combination therapy use in certain patients with multiple myeloma (Garnock-Jones, 2015) and is also being investigated for acute lymphocytic leukaemia and prostate cancer (Giles et al., 2006; Rathkopf et al., 2010). While vorinostat and panobinostat have previously been shown to inhibit the *in vitro* growth of *P. falciparum* (IC_{50} ~150 nM and ~20 nM, respectively (Patel et al., 2009)), romidepsin and belinostat have not previously been examined for anti-malarial activity. Panobinostat and belinostat have been shown to inhibit *T. b. brucei* growth (IC_{50} 1.6 μM and 2.2 μM , respectively (Carrillo et al., 2015)), however the activity of vorinostat and romidepsin have not been reported against this parasite species. To compare the *in vitro* anti-proliferative activities of these compounds against *Plasmodium* and *Trypanosoma* parasites and to begin to determine whether they have differential effects in parasites, we also profiled them for their effect on *P. falciparum* deacetylase inhibition and *in situ* hyperacetylation.

2. Materials and methods

2.1. Compounds

Suberoylanilide hydroxamic acid (SAHA; Sigma Aldrich, USA) and chloroquine (chloroquine diphosphate salt; Sigma Aldrich, USA) were prepared as 10 mM stock solutions in 100% DMSO. Belinostat (PXD101), romidepsin (FK228) and panobinostat (LBH589) were purchased from Selleck Chemicals and prepared as 20 mM stock solutions in 100% DMSO. Pentamidine, diminazene aceturate and puromycin were all purchased from Sigma Aldrich and 0.16, 9.7 and 9.2 mM stock solutions prepared in 100% DMSO.

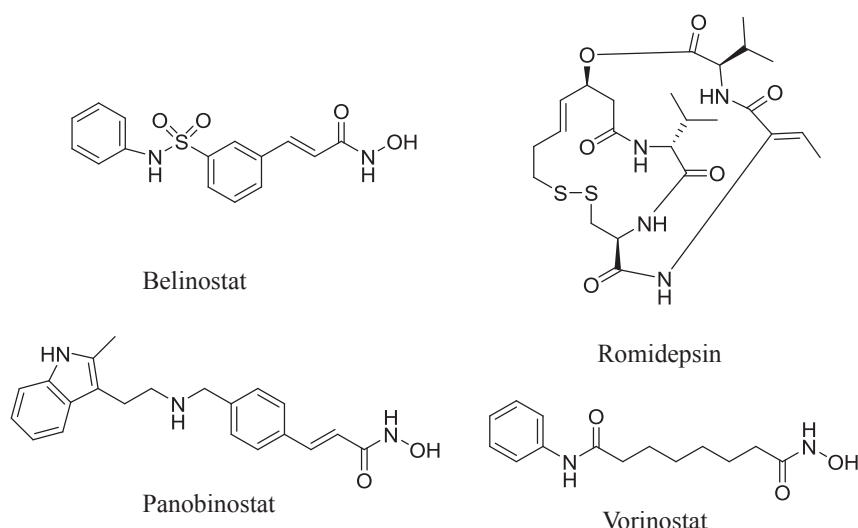


Fig. 1. Chemical structures of the hydroxamate HDAC inhibitors belinostat, panobinostat and vorinostat and the cyclic tetrapeptide HDAC inhibitor romidepsin.

2.2. *P. falciparum* growth inhibition assays

P. falciparum 3D7 (Walliker et al., 1987) and Dd2 (Wellemans et al., 1988) parasites were cultured *in vitro* with O + human erythrocytes in RPMI 1640 (Life Technologies) containing 10% heat inactivated human serum and 50 mg/L hypoxanthine. Parasites were cultured at 37 °C in 5% O₂ and 5% CO₂ in N₂. *In vitro* growth inhibition assays were assessed using the [³H] hypoxanthine incorporation method, essentially as previously described (Andrews et al., 2008). Briefly, synchronous ring-stage *P. falciparum* parasitized erythrocytes (0.25% parasitemia and 2.5% haematocrit) were incubated in 96-well plates with serial dilutions of test compounds or controls (concentrations starting from 0.5 to 2.5 µM) for 48 h at 37 °C in parasite gas mixture (5% O₂, 5% CO₂, and 90% N₂). Following addition of [³H] hypoxanthine (0.5 µCi/well) plates were incubated as above for a further 24 h. Assays were stopped by freezing at –20 °C. [³H]-hypoxanthine incorporation was determined by harvesting cells onto glass fibre filter mats and counting using a Perkin Elmer/Wallac Trilux 1450 MicroBeta scintillation counter. Percent inhibition of growth was compared to vehicle controls (0.5% DMSO) and 50% inhibitory concentrations calculated using linear interpolation of inhibition curves. Assays were carried out in triplicate wells, on three separate occasions with data presented as mean IC₅₀ (±standard deviation [SD]). Chloroquine and vorinostat were used in each assay as positive controls and an unpaired two-tailed *t* test was used for statistical analysis of differences in IC₅₀s.

2.3. *T. b. brucei* growth inhibition assays

T. b. brucei 427 bloodstream trypanosomes were cultured *in vitro* at 37 °C in 5% CO₂ with HMI-9 medium supplemented with 10% foetal bovine serum (FBS; Thermo Scientific). Compound activity was assessed using an Alamar blue® viability assay, as previously described (Sykes and Avery, 2009). Briefly, logarithmic phase *T. b. brucei* parasites (1200 cells/ml) were added to 384-well microtitre plates and incubated for 24 h at 37 °C in 5% CO₂. Parasites were subsequently exposed to serial dilutions of test compounds at concentrations ranging from 20.84 to 0.021 µM. Following incubation for 48 h at 37 °C in 5% CO₂, 10 µL of 0.49 mM resazurin (Sigma Aldrich, USA) prepared in HMI-9 media + 10% FCS was added to assay plates and incubated for a further 2 h at 37 °C in 5% CO₂ followed by 22 h at room temperature. Assay plates were read at 535 nm excitation/590 nm emission on an Envision® multiplate reader (PerkinElmer, USA). Data was analysed and IC₅₀ values calculated using the software GraphPad Prism 5. Pentamidine and diminazine aceturate were used as positive controls. *T. b. brucei*, the cause causative agent of Trypanosomiasis in cattle, closely resembles human infective *Trypanosoma* species and was used as a model for these studies. An unpaired two-tailed *t* test was used for statistical analysis.

2.4. NFF cytotoxicity assays

Neonatal foreskin fibroblast (NFF) cells were cultured in RPMI 1640 media supplemented with 10% foetal calf serum and 1% penicillin/streptomycin (Life Technologies, USA). Cells were seeded into 96 well plates (3000/well) and incubated for 24 h at 37 °C in 5% CO₂. Cells were then treated with compounds in a 2-fold dilution series with starting drug concentrations ranging from 200 µM to 10 nM. After 72 h drug exposure media was removed and plates were washed with PBS, 50 µL of 0.4% sulforhodamine B (Sigma Aldrich, USA) was added for 1 h prior to washing 3 times with 1% acetic acid. After tapping dry 100 µL 10 mM Tris base (unbuffered, pH > 9) was added to plates for a further 30 min. Assay results were

read at λ = 564 nm in a Synergy 2 plate reader (BioTek). Three independent assays were carried out, each in triplicate wells. IC₅₀ concentrations were calculated using linear interpolation of inhibition curves. Chloroquine and vorinostat were used in each assay as positive controls and an unpaired two-tailed *t* test was used for statistical analysis.

2.5. HEK293 cytotoxicity assays

HEK293 cells were cultured *in vitro* in DMEM medium supplemented with 10% FBS (Life Technologies) at 37 °C in 5% CO₂. Cells (72,727 cells/mL) were seeded in 384-well microtitre plates and incubated for 24 h at 37 °C in 5% CO₂ before being exposed to serial dilutions of test compounds at concentrations ranging from 41.68 to 0.021 µM for 48 h at 37 °C in 5% CO₂. Following drug exposure, 10 µL of 0.49 mM resazurin (Sigma Aldrich) prepared in DMEM media + 10% FCS was added to assay plates and incubated for 5 h at 37 °C in 5% CO₂ and then for 19 h at room temperature. Assay plates were read at λ = 535 nm excitation/590 nm emission on an Envision® multiplate reader (PerkinElmer, USA). Data were analysed and IC₅₀ values calculated using the software GraphPad Prism 5. Puromycin was included in each assay as a positive control and an unpaired two-tailed *t* test was used for statistical analysis.

2.6. Protein hyperacetylation assays

Synchronous trophozoite-stage 3D7 parasites (3–5% parasitemia, 5% haematocrit) were incubated with 3 × IC₅₀ concentrations of test compounds or controls for 3 h in a six-well plate. Controls included vehicle only (0.05% DMSO), chloroquine as a negative control and vorinostat as a positive HDAC inhibitor control. Parasite infected-erythrocytes were pelleted by centrifugation and lysed with 0.15% saponin. Following centrifugation, parasite pellets were washed extensively with 1 × PBS to remove haemoglobin and the washed pellets resuspended in 1 × SDS-PAGE loading dye. Following heat denaturation at 94 °C, proteins were analysed by SDS-PAGE and Western blot. Proteins were transferred to PVDF membrane and Western blotting carried out using Odyssey blocking buffer (Li-Cor Biosciences) according to the manufacturer's instructions. Anti-tetra-acetyl histone H4 (1:2000 dilution), anti-acetyl histone H3 lysine 9 (1:1000 dilution) and anti-acetyl histone H3 N-terminus (1:500 dilution) primary antibodies (Millipore) were used with IRDye 680 goat anti-rabbit secondary antibody (1:10,000 dilution) (Li-Cor Biosciences). Anti-RAP2 (1:1000 dilution) primary antibody was used with IRDye 800CW goat anti-mouse secondary antibody (1:10,000 dilution) (Li-Cor Biosciences) as a loading control. Anti-(pan) acetyl lysine (K103) monoclonal antibody (1:1000 dilution) (Cell Signalling Technology) was used with IRDye 800CW goat anti-mouse secondary antibody (Li-Cor Biosciences). All primary antibodies were incubated with the PVDF membranes overnight at 4 °C, followed by secondary antibody incubation at room temperature for 45 min. Membranes were imaged using Odyssey Classic (Li-Cor Biosciences). Following analysis with the first antibody, PVDF membranes were stripped at room temperature for 1 h in 25 mM glycine, 1% SDS, pH 2. Complete stripping was confirmed by imaging on an Odyssey Classic (Li-Cor Biosciences). Membranes were then re-probed with Anti-RAP2 primary antibody as a loading control, with analysis as above. Densitometry analysis was carried out using Image Studio Lite Version 3.1 software. Each sample was normalised to its respective RAP2 loading control probed and detected on the same membrane, and results expressed as the fold change in relative density compared to the DMSO vehicle control (with the control set to one).

2.7. Deacetylase activity assays

Nuclear extracts were prepared from trophozoite stage *P. falciparum* 3D7 parasites using a nuclear protein extraction kit (Merck Millipore), according to the manufacturer's instructions. Nuclear extracts, and recombinant PfHDAC1 (Sigma Aldrich) were assessed in deacetylase activity assays. Nuclear extracts (~0.3 mg/ml) were incubated with different concentrations (500–0.05 nM) of vorinostat, belinostat, panobinostat, romidepsin or trichostatin A (TSA; as an HDAC inhibitor control) in 96-well plates. For the PfHDAC1 deacetylase assays, PfHDAC1 (32 ng/ml) was incubated with 1 µM vorinostat, belinostat, panobinostat, romidepsin, TSA or vehicle only (0.5% DMSO) in 96-well plates. Ac-RGK(Ac)-AMC fluorogenic peptide substrate (R&D Systems) was added to each well and samples incubated at 37 °C for 1 h. Activator solution was then added, and samples incubated at room temperature for a further 10 min. Plates were then read in a Synergy 2 plate reader (BioTek; excitation: 350–380 nm, emission: 440–460 nm) within 60 min. HELA nuclear extracts (266.6 µg/ml) incubated with and without 1 µM TSA served as an assay control. Three independent assays were carried out for the 3D7 nuclear extract deacetylase assay and results are expressed as mean percent inhibition (±standard error of the mean [SEM]). 50% inhibitory concentrations were calculated using linear interpolation of inhibition curves with data presented as mean IC₅₀ (±SD). Two independent assays, each in duplicate, were carried out for the PfHDAC1 deacetylase assay with results expressed as percent inhibition of PfHDAC1 enzymatic activity (±SD) compared to DMSO vehicle controls.

3. Results

3.1. In vitro activity of HDAC inhibitors against *P. falciparum* asexual-stage parasites and bloodstream form *T. b. brucei*

The *in vitro* activity of HDAC inhibitors was assessed against two protozoan parasite species; drug sensitive (3D7) and drug resistant (Dd2) asexual *P. falciparum* parasites and bloodstream form *T. b. brucei* 427 parasites. Romidepsin and belinostat displayed nanomolar range activity against 3D7 and Dd2 *P. falciparum* lines (Table 1; IC₅₀ < 0.2 µM). The IC₅₀ of romidepsin was similar to that of vorinostat ($P > 0.05$) and significantly lower for belinostat versus vorinostat ($P = 0.01$). The IC₅₀'s of these compounds were, however, significantly higher than those of panobinostat ($P < 0.05$). The IC₅₀ values for vorinostat, panobinostat and the control anti-malarial drug, chloroquine, were consistent with previously published data (Patel et al., 2009; Sumanadasa et al., 2012). A comparison of *P. falciparum* activity data (IC₉₀) with the maximum plasma concentration (C_{max}) achieved in human subjects following administration of belinostat (C_{max} = 88.12–182.92 µM (Yeo et al., 2012)) or panobinostat (C_{max} = 0.07–1.97 µM (Prince et al., 2009)) indicates

that both of these drugs have activity against *P. falciparum* (IC₉₀ belinostat 0.12 µM; IC₉₀ panobinostat 0.01 µM) below clinically relevant concentrations (Fig. 2). Vorinostat (human plasma C_{max} = 0.43–2.98 µM (Rubin et al., 2006); *P. falciparum* IC₉₀ 0.24 µM) also has activity at, or below the clinically relevant range, while the activity of romidepsin against *P. falciparum* (IC₉₀ 0.18 µM) was within, but not below, the human C_{max} plasma range for this drug (C_{max} = 0.07–1.02 µM (Sandor et al., 2002)).

Next the activity of the anti-cancer HDAC inhibitors was tested against *T. b. brucei* 427 bloodstream trypanosomes. While *T. b. brucei* cannot infect primates, this species is genotypically very similar to the two human-infecting pathogenic species and is therefore a validated model for early phase drug discovery for Trypanosomiasis (e.g. (Faria et al., 2015)). Of the four HDAC inhibitors tested, the cyclic tetrapeptide pro-drug romidepsin was the most active compound (Table 1; IC₅₀ 0.035 µM), having significantly lower IC₅₀s (48–283-fold; $P < 0.0001$) than the other HDAC inhibitors which all have a hydroxamate zinc binding group. Belinostat, panobinostat and vorinostat were ~100-times less active against *T. b. brucei* than *P. falciparum* parasites. The *in vitro* activity of panobinostat and belinostat against *T. b. brucei* was similar to that reported in a previous study (Carrillo et al., 2015). When the anti-Trypanosomal activity (IC₉₀) of these compounds is compared with achievable plasma concentrations in human subjects following administration of these drugs (Fig. 2), vorinostat (*T. b. brucei* IC₉₀ 18.73 µM), panobinostat (*T. b. brucei* IC₉₀ 3.28 µM) and romidepsin (*T. b. brucei* IC₉₀ 0.10 µM) activity was all within, or above, the C_{max} range. Only the anti-Trypanosomal activity of belinostat (*T. b. brucei*; IC₉₀ 3.64 µM) fell below the C_{max} range of this drug (Fig. 2). These data comparing *T. b. brucei* IC₉₀'s with published *in vivo* C_{max} data (Fig. 2) are presented for comparison with *P. falciparum* data only. For the majority of patients diagnosed with African Trypanosomiasis, parasites are in the central nervous system not the peripheral circulation (reviewed in (Jones and Avery, 2013)). In addition, in this study *T. b. brucei* was used as an *in vitro* screening model and thus additional confirmatory studies with human infecting species need to be carried out in the future.

3.2. In vitro cytotoxicity of HDAC inhibitors against mammalian cell lines

To assess the *in vitro* selectivity of compounds for parasites versus mammalian cells, cytotoxicity assays were carried out against neonatal foreskin fibroblast (NFF) cells, using the previously published sulforhodamine B method (Skehan et al., 1990) and against HEK293 cells using the previously published Alamar blue® assay (Page et al., 1993). Belinostat and vorinostat demonstrated the greatest *P. falciparum*-specific selectivity against NFF cells (Table 1; SI 11–40 and 27–46, respectively). In contrast to the *P. falciparum* data, none of the HDAC inhibitors tested were

Table 1
In vitro anti-malarial plasmodial and anti-trypanosomal activity of anti-cancer HDAC inhibitors.

Compound	Mammalian cell IC ₅₀ (µM)		<i>P. falciparum</i> IC ₅₀ (µM)		<i>P. falciparum</i> SI ^b	<i>T. b. brucei</i> IC ₅₀ (µM)	<i>T. b. brucei</i> SI ^b
	NFF	HEK 293 ^a	3D7	Dd2			
Romidepsin	0.001 (±0.001)	<0.005	0.09 (±0.03)	0.13 (±0.04)	<1	0.035 (±0.01)	<1
Belinostat	2.37 (±1.61)	1.42 (±0.05)	0.06 (±0.02)	0.13 (±0.01)	11–40	2.61 (±0.21)	<1
Panobinostat	0.07 (±0.01)	0.18 (±0.03)	0.01 (±0.002)	0.03 (±0.04)	2–18	1.69 (±0.24)	<1
Vorinostat	5.50 (±1.31)	5.17 (±0.64)	0.12 (±0.06)	0.19 (±0.02)	27–46	9.03 (±1.81)	<1
Chloroquine	48.93 (±19.22)	nd	0.01 (±0.002)	0.08 (±0.01)	612–4893	9.92 (±2.39)	5
Pentamidine	nd	>0.69	nd	nd	nd	0.004 (±0.002)	>173
Diminazene aceturate	nd	>37	nd	nd	nd	0.06 µM (±0.002)	>617

^a Puromycin was the positive control for the HEK293 cytotoxicity assay (IC₅₀ 797.03 nM µM (±64.95)).

^b Selectivity Index – mammalian cell IC₅₀/*P. falciparum* IC₅₀ or *T. brucei* IC₅₀. Larger values indicate greater parasite selectivity; nd, not determined.

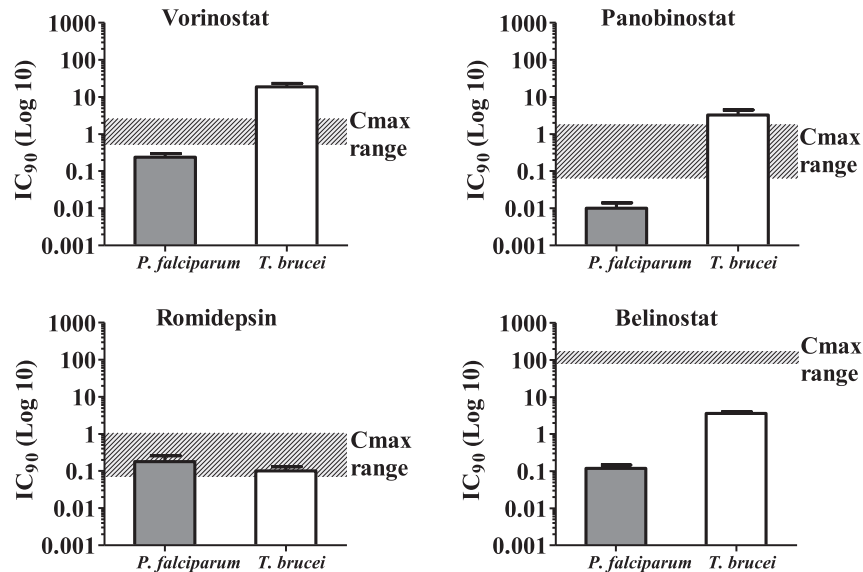


Fig. 2. Comparison of clinically achieved plasma concentrations of HDAC inhibitors with *P. falciparum* and *T. b. brucei* in vitro activity data. The C_{max} range of each compound (black and white stripes) was compared against the IC_{90} s of HDAC inhibitors against *P. falciparum* (grey bars) and *T. b. brucei* (white bars). The C_{max} range for vorinostat, administered to human subjects at 400 mg orally, once daily with food was 0.43–2.98 μ M (Rubin et al., 2006). The C_{max} range for belinostat, administered to patients at 600–1400 mg/m² over a 30 min intravenous infusion once on days 1–5 on a 21-day cycle, was 88.12–182.92 μ M (Yeo et al., 2012). The C_{max} range for romidepsin, administered at 1–24.9 mg/m² over a 4 h intravenous infusion on days 1 and 5 of a 21-day cycle, was 0.07–1.02 μ M (Sandor et al., 2002). The C_{max} range for panobinostat, administered by intravenous infusion and orally with doses ranging between 4.8 and 20 mg/m² and 15–80 mg, respectively, was 0.07–1.97 μ M (Prince et al., 2009).

selective for *T. b. brucei* parasites compared with normal mammalian cells (Table 1; SI < 1).

3.3. Activity studies against *P. falciparum* asexual-stage parasites

In order to determine whether romidepsin, belinostat and panobinostat cause similar biological effects on *P. falciparum* parasites as other anti-malarial HDAC inhibitors such as vorinostat (Dow et al., 2008; Sumanadasa et al., 2012), protein hyperacetylation assays were carried out. Trophozoite-stage *P. falciparum* 3D7 parasites were treated for 3 h with $\sim 3 \times IC_{50}$ concentrations of each compound. All assays included treatments with vorinostat as an HDAC inhibitor control, chloroquine as a negative control and vehicle only controls (0.05% DMSO). Equivalent loading between lanes was confirmed on the same membrane, following stripping, using a monoclonal antibody that recognises *P. falciparum* rhoptry associated protein 2 (RAP2; anti-1C3) (Baldi et al., 2000). As previously observed for vorinostat (Sumanadasa et al., 2012), using antibody that detects acetylated histone H4 (also known to cross-react with acetylated histone H2 forms; Millipore product manual) belinostat and romidepsin treatment caused hyperacetylation (Fig. 3A). In contrast treatment with panobinostat or chloroquine did not result in hyperacetylation compared to the vehicle control using this antibody (Fig. 3A). Vorinostat, belinostat and romidepsin all caused a > 1.3-fold increase in acetylation signal compared to the control (calculated as relative density, normalised to a loading control present on the same membrane), with romidepsin showing the greatest effect on acetylation detected using the anti-(tetra) acetyl H4 antibody (2.4-fold increase compared to control; Fig. 3A). In contrast, hyperacetylation was observed for all HDAC inhibitors using an antibody that recognises acetylation of the histone H3 lysine 9 residue (Fig. 3B). This antibody also appears to have some cross-reactivity, likely with another histone form. Vorinostat and romidepsin caused a ~ 5 – 6 -fold increase in the hyperacetylation using anti-histone H3 lysine 9 antibody as compared with the vehicle control. In addition, all HDAC inhibitors, except panobinostat, caused hyperacetylation using an antibody

that recognises H3 N-terminal acetylation (Fig. 3C). Vorinostat caused a 6-fold increase in acetylation using anti-N-terminal H3 antibody compared to the vehicle control. All HDAC inhibitors also caused hyperacetylation of a non-histone protein of ~ 80 – 90 kDa detected using an antibody that recognises pan-acetyl lysine residues, giving a 12–27-fold increase in relative density compared to the control (Fig. 3D). Together these data indicate that all four HDAC inhibitors hyperacetylate both histone and non-histone proteins in *P. falciparum*, although there do appear to be differences in the levels of acetylation observed for different histones.

To confirm that these HDAC inhibitors target *P. falciparum* HDAC activity, their capacity to inhibit deacetylase activity of *P. falciparum* nuclear extracts and recombinant PfHDAC1 was assessed utilizing Ac-RGK(Ac)-AMC fluorogenic peptide substrate (R&D Systems; Fig. 4 and Table 2). All HDAC inhibitors, including the hydroxamate HDAC inhibitor assay control TSA, inhibited deacetylase activity of parasite nuclear extracts and recombinant PfHDAC1. The calculated IC_{50} of TSA, panobinostat and romidepsin was < 4 nM for deacetylase inhibitory activity of *P. falciparum* 3D7 nuclear extracts (Table 2), while belinostat and vorinostat were less active, with IC_{50} values ~ 55 – 373 times higher than the other compounds (Table 2; IC_{50} 214.7 nM and 335.5 nM, respectively). Panobinostat, together with the assay control TSA, inhibited $\sim 100\%$ of the enzymatic activity of recombinant PfHDAC1 at 1 μ M. The percentage inhibition of vorinostat, romidepsin and belinostat ranged from 48 to 79%. Although romidepsin had the lowest deacetylase IC_{50} (0.9 (± 0.8) nM) this compound showed the least inhibition of PfHDAC1 (48.3% \pm 39.3%; Table 1). However, as there was variation between experiments it is not possible to draw any conclusions about possible positive or negative correlations from these data. Together with the hyperacetylation assay data, these data support these anticancer HDAC inhibitors as targeting PfHDAC activity in *P. falciparum*.

4. Discussion

Among different approaches used for anti-parasitic drug discovery, the ‘piggyback’ approach, which involves using previously

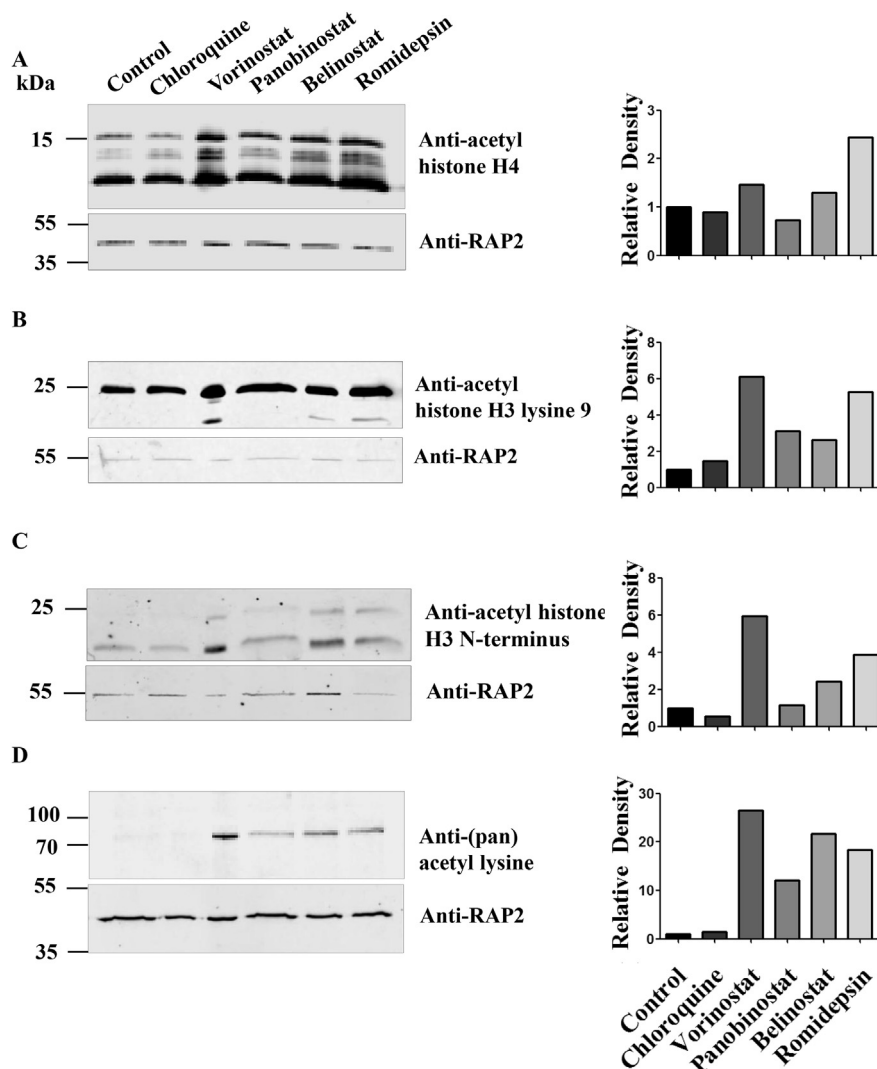


Fig. 3. Hyperacetylation of *P. falciparum* proteins by HDAC inhibitors. Trophozoite-stage *P. falciparum* 3D7 parasites were treated with $3 \times \text{IC}_{50}$ of different HDAC inhibitors (vorinostat, panobinostat, belinostat or romidepsin) or controls (DMSO vehicle or chloroquine) for 3 h followed by preparation of protein lysates and Western blot analysis using rabbit antibodies specific to tetra-acetylated H4 (A), histone H3 lysine 9 (B), histone H3 lysine acetylated N-terminus (C), or (pan) acetyl lysine (D). Each membrane was stripped and re-probed with anti-RAP2 monoclonal antibody to allow loading on the same gel to be ascertained. Membranes were imaged using Odyssey Classic (Li-Cor Biosciences). Densitometry analysis was performed and each sample was normalised to the RAP2 loading control. Results are expressed as the fold change in relative density compared to the control, set to one.

evaluated or approved drugs as therapeutic starting points for other diseases, has gained greater attention in recent years (reviewed in (Nwaka and Hudson, 2006; Caffrey and Steverding, 2008; Andrews et al., 2014)). Piggy-backing onto drug discovery efforts for cancer and other diseases, in particular the development of next generation compounds with targeted selectivity, is also a strategy in the identification of new drug leads for parasitic diseases (reviewed in (Andrews et al., 2014)).

In this study we investigated the *in vitro* anti-Plasmodial and anti-Trypanosomal activity of HDAC inhibitors that are clinically approved, and undergoing clinical investigation, for human cancer therapy. Vorinostat, belinostat, panobinostat and romidepsin all exhibit *in vitro* growth inhibitory activity against asexual-stage *P. falciparum* parasites in the nM nanomolar range. While this is the first report of belinostat and romidepsin having asexual stage anti-malarial activity, panobinostat and romidepsin have recently been shown to have activity against sexual stage *P. falciparum* gametocytes (IC_{50} 0.94 μM and 0.64 μM , respectively) (Sun et al., 2014) with > 10-fold selectivity for gametocytes compared to the

mammalian cell line HepG2 (Sun et al., 2014). Together with data presented here, this suggests that these two compounds can target both asexual and gametocyte intraerythrocytic stage parasites. This also supports our own recent finding that vorinostat and TSA target early (IC_{50} 1.41 μM and 0.09 μM , respectively) and late (IC_{50} 0.81 μM and 0.07 μM , respectively) stage gametocytes (Trenholme et al., 2014). Combined with studies showing that exoerythrocytic (liver stage) *Plasmodium* parasites are also sensitive to certain HDAC inhibitors (Sumanadasa et al., 2012; Hansen et al., 2014b), these data indicate that HDAC inhibitors are a promising class of compounds with broad-stage anti-Plasmodial activity.

Like the control HDAC inhibitor vorinostat (SI = 27–46; Table 1), belinostat and panobinostat were found to have modest selectivity for *P. falciparum* compared with the mammalian cell lines examined (SI = 11–40 and 2–18, respectively; Table 1). In comparison to the control antimalarial drug chloroquine (SI 612–4839; Table 1) this level of selectivity is low, comparable to an early stage drug-lead. Although we are not proposing that these anti-cancer HDAC inhibitors be used for malaria therapy *per se*, it

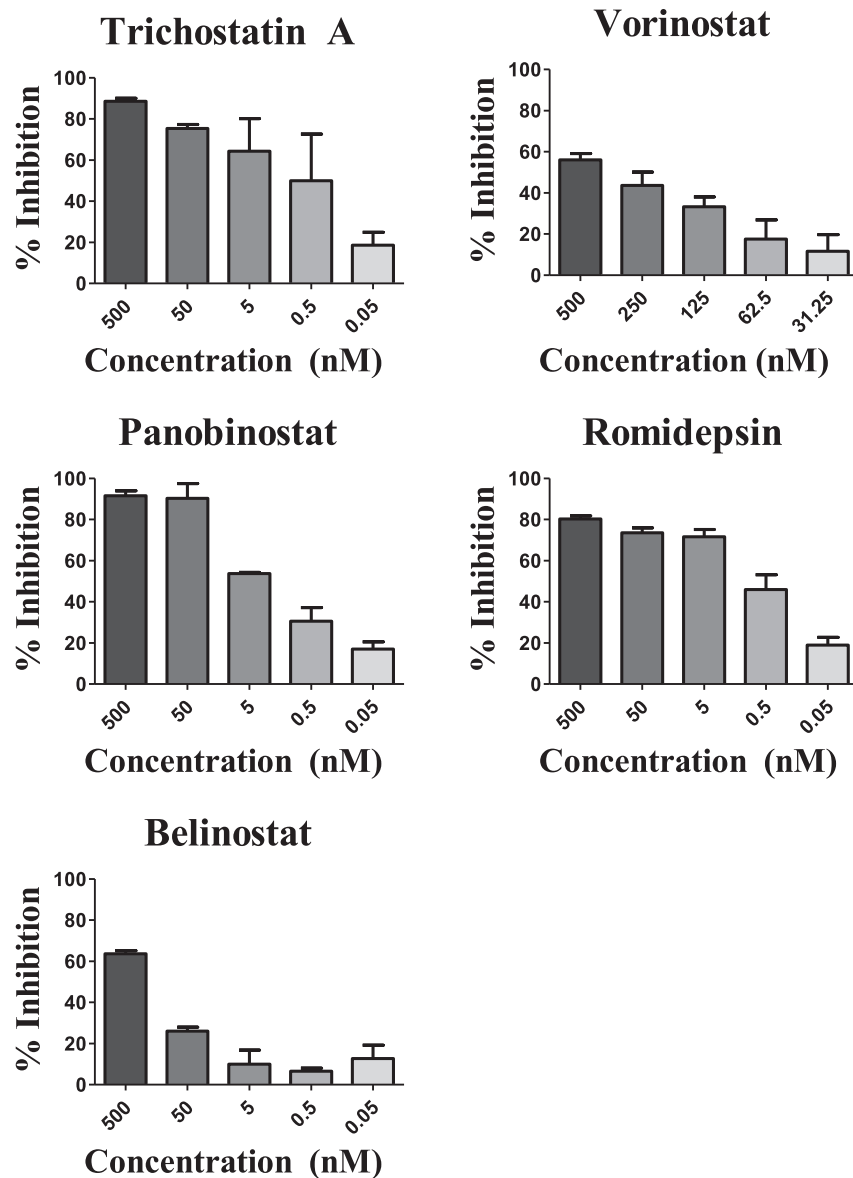


Fig. 4. Inhibition of deacetylase activity in *P. falciparum* nuclear extracts. The HDAC inhibitors vorinostat, belinostat, panobinostat, romidepsin and TSA were assessed for the inhibition of deacetylase activity in nuclear extracts prepared from trophozoite-stage *P. falciparum* 3D7 parasites using the Ac-RGK(Ac)-AMC fluorogenic peptide substrate. The mean percent inhibition (\pm standard error of the mean [SEM]) for three independent assays is shown.

is interesting to note that the activity of the compounds was within or below the clinically relevant range (Fig. 2). This is promising in terms of future development of more potent and parasite-selective compounds that may have improved pharmacokinetic profiles suited to malaria therapy. Thus the piggyback approach has had value here in providing new clues to HDAC

inhibitor drug development for malaria based on existing HDAC-targeting anti-cancer drugs.

In contrast to the findings for *P. falciparum*, romidepsin, the only cyclic tetrapeptide examined in this study, was also the only HDAC inhibitor with sub-micromolar activity against *T. b. brucei* blood-stream parasite forms (IC_{50} 0.035 μ M; Table 1). Unfortunately, this

Table 2

Deacetylase activity of HDAC inhibitors against *P. falciparum* nuclear extracts and recombinant PfHDAC1.

Compound	<i>P. falciparum</i> nuclear extract IC_{50} (nM) ^a	PfHDAC1 % inhibition at 1 μ M ^b
TSA	3.9 \pm 3.4	98.9 \pm 1.5
Vorinostat	335.5 \pm 145.5	59.8 \pm 12.7
Panobinostat	3.3 \pm 0.7	100 \pm 0
Romidepsin	0.9 \pm 0.8	48.3 \pm 39.3
Belinostat	214.7 \pm 15.3	78.5 \pm 4.7

^a IC_{50} values are mean (\pm SD) of three independent dose response experiments (Fig. 4).

^b Mean % inhibition (\pm SD) of two independent assays.

compound exhibited no parasite-specific selectivity ($SI < 1$; Table 1). Belinostat, panobinostat and vorinostat were also found to be non-selective for *T. b. brucei* parasites when compared with activity against normal mammalian cells (Table 1). While other studies examining the activity and selectivity of HDAC inhibitors against *Trypanosoma* parasites are limited, they are generally in line with data from the present study with compounds tested to date all having poor selectivity for this parasite species. The cyclic tetrapeptide HDAC inhibitor apicidin has previously been shown to have poor and non-selective activity against *Trypanosoma brucei* parasites (Murray et al., 2001), a lack of selectivity also being observed for *P. falciparum* (Darkin-Rattray et al., 1996). The only other HDAC inhibitors tested to date, experimental hydroxamate-derived compounds with sub- μ M activity against bloodstream *T. b. brucei* 427 also inhibited HDACs in mammalian cells (HeLa) with similar potency, although comments on whole cell selectivity cannot be made as normal mammalian cell proliferation was not investigated (Kelly et al., 2012).

In addition to investigating the growth inhibitory activity of HDAC inhibitors against *P. falciparum*, in this study we also profiled their action on the parasite using three different approaches – examination of *in situ* hyperacetylation changes in response to compound exposure (Fig. 3), inhibition of *P. falciparum* nuclear protein lysates, and inhibition of recombinant PfHDAC1 (Table 2). These studies were not conducted with *Trypanosoma* parasites because, unlike *P. falciparum* which contains canonical histones that are highly conserved with those of other eukaryotes (as well as four variants; (Miao et al., 2006)), *T. brucei* histones are divergent, in particular at their posttranslationally modified N-termini (Strahl and Allis, 2000; Alsford and Horn, 2004; Mandava et al., 2007). This means that commercial acetyl lysine antibodies, which cross-react with *P. falciparum* canonical histones, cannot be used for *T. brucei*. In *in situ* hyperacetylation assays with *P. falciparum* parasites, belinostat, panobinostat and romidepsin were shown to hyperacetylate both histone and non-histone proteins, consistent with previous data for other HDAC inhibitors including vorinostat and SB939 (Sumanadasa et al., 2012; Hansen et al., 2014a). All four HDAC inhibitors caused hyperacetylation of a ~80 kDa protein when probed with an antibody that is pan-reactive for acetylated lysines. We have previously shown that this protein is hyperacetylated by SAHA, SB939 and other hydroxamates (Sumanadasa et al., 2012). Although not yet identified, the protein may be *P. falciparum* heat shock protein 90 (PfHSP90) based on data showing that this protein is detected in parasite lysate complexes treated with TSA and probed with the same antibody (Pallavi et al., 2010). Vorinostat, belinostat and romidepsin all appeared to have similar profiles in hyperacetylating proteins detected by histone H3 and H4 antibodies, with vorinostat causing the greatest effect using anti-histone H3 antibody (~6-fold increase in density compared to the control). Acetylation detected by anti-(tetra) acetyl histone H4 antibodies on the other hand was affected most by romidepsin, which resulted in a 2.4-fold increase in relative density. Interestingly panobinostat was the only HDAC inhibitor that did not show hyperacetylation using both histone H3 and H4 antibodies in the Western blot analysis. Panobinostat was found to cause hyperacetylation using an anti-histone H3 lysine 9 antibody, but did not increase acetylation as detected by anti-histone H3 N-terminus antibody or anti-(tetra) acetyl H4 antibody. It may be that this compound acts differently to the other HDACs, however although all compounds were tested at $3 \times IC_{50}$ concentrations to try to normalize effects, subtle differences in compound uptake and target access also need to be considered. Overall these data, which represent the first study aimed at trying to detect differences in acetylation profiles in *P. falciparum* in response to HDAC inhibitors, indicate that there may be compound-specific differences in HDAC

inhibitor action, possibly due to the targeting of different PfHDAC isoforms. As discussed in greater detail below, additional studies will be required as there may also be differences in uptake and targeting of compounds and this may also impact on mechanistic effects.

All of the HDAC inhibitors examined in this study were shown to inhibit deacetylase activity in *P. falciparum* nuclear extracts prepared from trophozoite-stage parasites. The highest inhibition was seen for romidepsin (Table 2; IC_{50} 0.9 nM). TSA and panobinostat displayed similar potencies with IC_{50} s ~3–4 nM for nuclear extract deacetylase inhibition. Vorinostat and belinostat had the highest *P. falciparum* nuclear extract deacetylase IC_{50} s (Table 2; 335.5 nM and 214.7 nM, respectively), ~55–394-fold higher than those of romidepsin, TSA, and panobinostat. Similar levels of enzyme inhibition have previously been reported for these two compounds against different human HDAC isoforms (HDAC1, 2, 3, 4, 6, 7, 8 and 9) (Khan et al., 2008). However, unlike this study where similar *P. falciparum* growth inhibition was obtained (Table 1), in proliferation inhibition studies with mammalian cells, belinostat has been shown to be up to 11-fold more active than vorinostat (Khan et al., 2008; Andrews et al., 2012a; Sumanadasa et al., 2012; Parker et al., 2013). While it is tempting to try to correlate the nuclear extract deacetylase inhibition data with the *P. falciparum* growth inhibition data generated in this study, differences in compound uptake, access to cellular targets following uptake, drug stability, and variations in off-target effects will impact activity and make these direct comparisons difficult.

A major limitation of the study of *P. falciparum* HDACs is the lack of availability of recombinant HDAC enzymes. Only one *P. falciparum* HDAC enzyme is currently available, PfHDAC1, and its use is compromised by high cost and poor purity. Given these limitations the HDAC inhibitors examined in this study were tested for activity against recombinant PfHDAC1, but at a single concentration. Data generated showed that all compounds inhibited PfHDAC1 by at least ~50% when tested at 1 μ M (Table 2). As discussed above, vorinostat and panobinostat have previously been shown to inhibit recombinant PfHDAC1 enzymatic activity with IC_{50} 59 nM and 1.8 nM, respectively (Patel et al., 2009). These data indicate that this enzyme may be a target in *P. falciparum* parasites. However, as other PfHDAC isoforms are not presently available, it remains to be determined whether other HDACs are also targeted, or differentially targeted, by the different compounds. Likewise, while the presence of PfHDAC1 was confirmed in nuclear lysates by Western blot (data not shown) using a polyclonal anti-PfHDAC1 antibody (Trenholme et al., 2014), antibodies to the other class I/II HDACs are not yet available so we do not know which HDAC isoforms are expressed in nuclear extracts or their relative abundance.

In summary, this study reveals that four HDAC inhibitors clinically approved for the treatment of cancer have dose-dependent anti-parasitic activity against *Plasmodium* and *Trypanosoma* parasites, causative agents of malaria and trypanosomiasis. Although the profile of the inhibitors examined in this study, in particular their relatively low selectivity for parasites versus mammalian cells, does not support their use clinically for malaria or trypanosomiasis, our data do indicate that they may be useful starting points for further development of new compounds that may have more suitable anti-parasitic profiles. In particular, it seems that alternative approaches need to be taken to identify more parasite-selective compounds. HDAC inhibitor mode of action studies are currently limited by reagent availability, however, these studies are a high priority if the activity of this class of inhibitors is to be exploited for malaria and other parasite species. In this paper we also present intriguing data that suggests there are differences in how different HDAC inhibitors target HDAC enzymes in malaria parasites and that

they may target different HDAC isoforms differentially. This raises the exciting possibility of the identification of isoenzyme selective anti-malarial HDAC inhibitors and their development into both stage-specific and pan-stage inhibitors of malaria parasites.

Acknowledgements

We thank the Australian Research Council (FT0991213 to KTA) and Australian National Health and Medical Research Council (APP1074016 and APP1093378 to KTA and DPF; APP1025581 to VMA; APP1027369 to DPF) for research and fellowship support and Griffith University for scholarship support (GUIPRS and GUPRS to JAE). We acknowledge the Australian Red Cross Blood Service for the provision of human blood and sera. This project was carried out as part of the A-PARADDISE program funded under the *European Union's Seventh Framework Programme* (to KTA and DPF).

References

- Alonso, V.L., Serra, E.C., 2012. Lysine acetylation: elucidating the components of an emerging global signaling pathway in trypanosomes. *J. Biomed. Biotechnol.* 2012, 452934.
- Alsford, S., Horn, D., 2004. Trypanosomatid histones. *Mol. Microbiol.* 53, 365–372.
- Andrews, K.T., Fisher, G., Skinner-Adams, T.S., 2014. Drug repurposing and human parasitic protozoan diseases. *Int. J. Parasitol.-Drugs Drug Resist.* 4, 95–111.
- Andrews, K.T., Haque, A., Jones, M.K., 2012a. HDAC inhibitors in parasitic diseases. *Immunol. Cell. Biol.* 90, 66–77.
- Andrews, K.T., Tran, T.N., Fairlie, D.P., 2012b. Towards histone deacetylase inhibitors as new antimalarial drugs. *Curr. Pharm. Des.* 18, 3467–3479.
- Andrews, K.T., Tran, T.N., Lucke, A.J., Kahnberg, P., Le, G.T., Boyle, G.M., Gardiner, D.L., Skinner-Adams, T.S., Fairlie, D.P., 2008. Potent antimalarial activity of histone deacetylase inhibitor analogues. *Antimicrob. Agents Chemother.* 52, 1454–1461.
- Aurrecoechea, C., Brestelli, J., Brunk, B.P., Dommer, J., Fischer, S., Gajria, B., Gao, X., Gingle, A., Grant, G., Harb, O.S., Heiges, M., Innamorato, F., Iodice, J., Kissinger, J.C., Kraemer, E., Li, W., Miller, J.A., Nayak, V., Pennington, C., Pinney, D.F., Roos, D.S., Ross, C., Stoekert Jr., C.J., Treatman, C., Wang, H., 2009. PlasmoDB: a functional genomic database for malaria parasites. *Nucleic Acids Res.* 37, D539–D543.
- Azzi, A., Cosseau, C., Grunau, C., 2009. *Schistosoma mansoni*: developmental arrest of miracidia treated with histone deacetylase inhibitors. *Exp. Parasitol.* 121, 288–291.
- Baldi, D.L., Andrews, K.T., Waller, R.F., Roos, D.S., Howard, R.F., Crabb, B.S., Cowman, A.F., 2000. RAP1 controls rhoptry targeting of RAP2 in the malaria parasite *Plasmodium falciparum*. *EMBO J.* 19, 2435–2443.
- Byrd, J.C., Marcucci, G., Parthun, M.R., Xiao, J.J., Klisovic, R.B., Moran, M., Lin, T.S., Liu, S., Sklenar, A.R., Davis, M.E., Lucas, D.M., Fischer, B., Shank, R., Tejaswi, S.L., Binkley, P., Wright, J., Chan, K.K., Grever, M.R., 2005. A phase 1 and pharmacodynamic study of depsipeptide (FK228) in chronic lymphocytic leukemia and acute myeloid leukemia. *Blood* 105, 959–967.
- Caffrey, C.R., Steverding, D., 2008. Recent initiatives and strategies to developing new drugs for tropical parasitic diseases. *Expert Opin. Drug Discov.* 3, 173–186.
- Campas-Moya, C., 2009. Romidepsin for the treatment of cutaneous T-cell lymphoma. *Drugs Today (Barc)* 45, 787–795.
- Carrillo, A.K., Guiguemde, W.A., Guy, R.K., 2015. Evaluation of histone deacetylase inhibitors (HDACi) as therapeutic leads for human African trypanosomiasis (HAT). *Bioorg. Med. Chem.* <http://dx.doi.org/10.1016/j.bmc.2014.12.066>.
- Coiffier, B., Pro, B., Prince, H.M., Foss, F., Sokol, L., Greenwood, M., Caballero, D., Borchmann, P., Morschhauser, F., Wilhelm, M., Pinter-Brown, L., Padmanabhan, S., Shustov, A., Nichols, J., Carroll, S., Balser, J., Balser, S.B., Horwitz, S., 2012. Results from a pivotal, open-label, phase II study of romidepsin in relapsed or refractory peripheral T-cell lymphoma after prior systemic therapy. *J. Clin. Oncol.* 30, 631–636.
- Coleman, B.L., Skillman, K.M., Jiang, R.H., Childs, L.M., Altenhofen, L.M., Ganter, M., Leung, Y., Goldowitz, I., Kafack, B.F., Marti, M., Llinas, M., Buckee, C.O., Duraisingh, M.T., 2014. A *Plasmodium falciparum* histone deacetylase regulates antigenic variation and gametocyte conversion. *Cell. Host Microbe* 16, 177–186.
- Darkin-Rattray, S.J., Gurnett, A.M., Myers, R.W., Dulski, P.M., Crumley, T.M., Allocco, J.J., Cannova, C., Meinke, P.T., Colletti, S.L., Bednarek, M.A., Singh, S., Goetz, M.A., Dombrowski, A.W., Polishook, J.D., Schmatz, D.M., 1996. Apicidin: a novel antiprotozoal agent that inhibits parasite histone deacetylase. *Proc. Natl. Acad. Sci. U. S. A.* 93, 13143–13147.
- De Ruijter, A.J., Van Gennip, A.H., Caron, H.N., Kemp, S., Van Kuilenburg, A.B., 2003. Histone deacetylases (HDACs): characterization of the classical HDAC family. *Biochem. J.* 370, 737–749.
- Dow, G.S., Chen, Y., Andrews, K.T., Caridha, D., Gerena, L., Gettayacamin, M., Johnson, J., Li, Q., Melendez, V., Obaldia 3rd, N., Tran, T.N., Kozikowski, A.P., 2008. Antimalarial activity of phenylthiazolyl-bearing hydroxamate-based histone deacetylase inhibitors. *Antimicrob. Agents Chemother.* 52, 3467–3477.
- Dubois, F., Cabby, S., Oger, F., Cosseau, C., Capron, M., Grunau, C., Dissous, C., Pierce, R.J., 2009. Histone deacetylase inhibitors induce apoptosis, histone hyperacetylation and up-regulation of gene transcription in *Schistosoma mansoni*. *Mol. Biochem. Parasitol.* 168, 7–15.
- Faria, J., Moraes, C.B., Song, R., Pascoalino, B.S., Lee, N., Siqueira-Neto, J.L., Cruz, D.J., Parkinson, T., Ioset, J.R., Cordeiro-Da-Silva, A., Freitas-Junior, L.H., 2015. Drug discovery for human African trypanosomiasis: identification of novel scaffolds by the newly developed HTS SYBR Green assay for *Trypanosoma brucei*. *J. Biomol. Screen* 20, 70–81.
- Freitas-Junior, L.H., Hernandez-Rivas, R., Ralph, S.A., Montiel-Condado, D., Ruvalcaba-Salazar, O.K., Rojas-Meza, A.P., Mancio-Silva, L., Leal-Silvestre, R.J., Gontijo, A.M., Shorte, S., Scherf, A., 2005. Telomeric heterochromatin propagation and histone acetylation control mutually exclusive expression of antigenic variation genes in malaria parasites. *Cell* 121, 25–36.
- Garcia-Manero, G., Yang, H., Bueso-Ramos, C., Ferrajoli, A., Cortes, J., Wierda, W.G., Faderl, S., Koller, C., Morris, G., Rosner, G., Loboda, A., Fantin, V.R., Randolph, S.S., Hardwick, J.S., Reilly, J.F., Chen, C., Ricker, J.L., Secrist, J.P., Richon, V.M., Frankel, S.R., Kantarjian, H.M., 2008. Phase 1 study of the histone deacetylase inhibitor vorinostat (suberoylanilide hydroxamic acid [SAHA]) in patients with advanced leukemias and myelodysplastic syndromes. *Blood* 111, 1060–1066.
- Garnock-Jones, K.P., 2015. Panobinostat: first global approval. *Drugs* 75, 695–704. <http://dx.doi.org/10.1007/s40265-015-0388-8>.
- Giles, F., Fischer, T., Cortes, J., Garcia-Manero, G., Beck, J., Ravandi, F., Masson, E., Rae, P., Laird, G., Sharma, S., Kantarjian, H., Dugan, M., Albitar, M., Bhalla, K., 2006. A phase I study of intravenous LBH589, a novel cinnamic hydroxamic acid analogue histone deacetylase inhibitor, in patients with refractory hematologic malignancies. *Clin. Cancer Res.* 12, 4628–4635.
- Grant, S., Easley, C., Kirkpatrick, P., 2007. Vorinostat. *Nat. Rev. Drug Discov.* 6, 21–22.
- Hansen, F.K., Skinner-Adams, T.S., Duffy, S., Marek, L., Sumanadasa, S.D., Kuna, K., Held, J., Avery, V.M., Andrews, K.T., Kurz, T., 2014a. Synthesis, antimalarial properties, and SAR studies of alkoxyurea-based HDAC inhibitors. *ChemMedChem* 9, 665–670.
- Hansen, F.K., Sumanadasa, S.D., Stenzel, K., Duffy, S., Meister, S., Marek, L., Schmetter, R., Kuna, K., Hamacher, A., Mordmuller, B., Kassack, M.U., Winzler, E.A., Avery, V.M., Andrews, K.T., Kurz, T., 2014b. Discovery of HDAC inhibitors with potent activity against multiple malaria parasite life cycle stages. *Eur. J. Med. Chem.* 82, 204–213.
- Ingram, A.K., Horn, D., 2002. Histone deacetylases in *Trypanosoma brucei*: two are essential and another is required for normal cell cycle progression. *Mol. Microbiol.* 45, 89–97.
- Jones, A.J., Avery, V.M., 2013. Whole-organism high-throughput screening against *Trypanosoma brucei*. *Expert Opin. Drug Discov.* 8, 495–507.
- Kelly, J.M., Taylor, M.C., Horn, D., Loza, E., Kalvinsh, I., Bjorkling, F., 2012. Inhibitors of human histone deacetylase with potent activity against the African trypanosome *Trypanosoma brucei*. *Bioorg. Med. Chem. Lett.* 22, 1886–1890.
- Khan, N., Jeffers, M., Kumar, S., Hackett, C., Boldog, F., Khramtsov, N., Qian, X., Mills, E., Berghs, S.C., Carey, N., Finn, P.W., Collins, L.S., Tumber, A., Ritchie, J.W., Jensen, P.B., Lichenstein, H.S., Sehested, M., 2008. Determination of the class and isoform selectivity of small-molecule histone deacetylase inhibitors. *Biochem. J.* 409, 581–589.
- Khan, O., La Thangue, N.B., 2012. HDAC inhibitors in cancer biology: emerging mechanisms and clinical applications. *Immunol. Cell. Biol.* 90, 85–94.
- Mai, A., Cerbara, I., Valente, S., Massa, S., Walker, L.A., Tekwani, B.L., 2004. Antimalarial and antileishmanial activities of aroyl-pyrrolyl-hydroxamides, a new class of histone deacetylase inhibitors. *Antimicrob. Agents Chemother.* 48, 1435–1436.
- Malmquist, N.A., Moss, T.A., Mecheri, S., Scherf, A., Fuchter, M.J., 2012. Small-molecule histone methyltransferase inhibitors display rapid antimalarial activity against all blood stage forms in *Plasmodium falciparum*. *Proc. Natl. Acad. Sci. U. S. A.* 109, 16708–16713.
- Mandava, V., Fernandez, J.P., Deng, H., Janzen, C.J., Hake, S.B., Cross, G.A., 2007. Histone modifications in *Trypanosoma brucei*. *Mol. Biochem. Parasitol.* 156, 41–50.
- Mariadason, J.M., 2008. HDACs and HDAC inhibitors in colon cancer. *Epigenetics* 3, 28–37.
- Miao, J., Fan, Q., Cui, L., Li, J., Li, J., Cui, L., 2006. The malaria parasite *Plasmodium falciparum* histones: organization, expression, and acetylation. *Gene* 369, 53–65.
- Modesitt, S.C., Sill, M., Hoffman, J.S., Bender, D.P., Gynecologic Oncology, G., 2008. A phase II study of vorinostat in the treatment of persistent or recurrent epithelial ovarian or primary peritoneal carcinoma: a gynecologic oncology group study. *Gynecol. Oncol.* 109, 182–186.
- Molife, L.R., Attard, G., Fong, P.C., Karavasilis, V., Reid, A.H., Patterson, S., Riggs Jr., C.E., Higano, C., Stadler, W.M., McCulloch, W., Dearnaley, D., Parker, C., De Bono, J.S., 2010. Phase II, two-stage, single-arm trial of the histone deacetylase inhibitor (HDACi) romidepsin in metastatic castration-resistant prostate cancer (CRPC). *Ann. Oncol.* 21, 109–113.
- Murray, P.J., Kranz, M., Ladlow, M., Taylor, S., Berst, F., Holmes, A.B., Keavey, K.N., Jaxa-Chamiec, A., Seale, P.W., Stead, P., Upton, R.J., Croft, S.L., Clegg, W., Elsegood, M.R., 2001. The synthesis of cyclic tetrapeptide analogues of the antiprotozoal natural product apicidin. *Bioorg. Med. Chem. Lett.* 11, 773–776.
- Nwaka, S., Hudson, A., 2006. Innovative lead discovery strategies for tropical diseases. *Nat. Rev. Drug Discov.* 5, 941–955.
- Page, B., Page, M., Noel, C., 1993. A new fluorometric assay for cytotoxicity measurements *in-vitro*. *Int. J. Oncol.* 3, 473–476.

- Pallavi, R., Roy, N., Nageshan, R.K., Talukdar, P., Pavithra, S.R., Reddy, R., Venketesh, S., Kumar, R., Gupta, A.K., Singh, R.K., Yadav, S.C., Tatu, U., 2010. Heat shock protein 90 as a drug target against protozoan infections: biochemical characterization of HSP90 from *Plasmodium falciparum* and *Trypanosoma evansi* and evaluation of its inhibitor as a candidate drug. *J. Biol. Chem.* 285, 37964–37975.
- Parker, J.P., Nimir, H., Griffith, D.M., Duff, B., Chubb, A.J., Brennan, M.P., Morgan, M.P., Egan, D.A., Marmion, C.J., 2013. A novel platinum complex of the histone deacetylase inhibitor belinostat: rational design, development and *in vitro* cytotoxicity. *J. Inorg. Biochem.* 124, 70–77.
- Patel, V., Mazitschek, R., Coleman, B., Nguyen, C., Urgaonkar, S., Cortese, J., Barker, R.H., Greenberg, E., Tang, W., Bradner, J.E., Schreiber, S.L., Duraisingh, M.T., Wirth, D.F., Clardy, J., 2009. Identification and characterization of small molecule inhibitors of a class I histone deacetylase from *Plasmodium falciparum*. *J. Med. Chem.* 52, 2185–2187.
- Patil, V., Guerrant, W., Chen, P.C., Gryder, B., Benicewicz, D.B., Khan, S.I., Tekwani, B.L., Oyeler, A.K., 2010. Antimalarial and antileishmanial activities of histone deacetylase inhibitors with triazole-linked cap group. *Bioorg. Med. Chem.* 18, 415–425.
- Prince, H.M., Bishton, M.J., Johnstone, R.W., 2009. Panobinostat (LBH589): a potent pan-deacetylase inhibitor with promising activity against hematologic and solid tumors. *Future Oncol.* 5, 601–612.
- Prince, H.M., Dickinson, M., 2012. Romidepsin for cutaneous T-cell lymphoma. *Clin. Cancer Res.* 18, 3509–3515.
- Rathkopf, D., Wong, B.Y., Ross, R.W., Anand, A., Tanaka, E., Woo, M.M., Hu, J., Dzik-Jurasz, A., Yang, W., Scher, H.I., 2010. A phase I study of oral panobinostat alone and in combination with docetaxel in patients with castration-resistant prostate cancer. *Cancer Chemother. Pharmacol.* 66, 181–189.
- Rubin, E.H., Agrawal, N.G., Friedman, E.J., Scott, P., Mazina, K.E., Sun, L., Du, L., Ricker, J.L., Frankel, S.R., Gottesdiener, K.M., Wagner, J.A., Iwamoto, M., 2006. A study to determine the effects of food and multiple dosing on the pharmacokinetics of vorinostat given orally to patients with advanced cancer. *Clin. Cancer Res.* 12, 7039–7045.
- Sandor, V., Bakke, S., Robey, R.W., Kang, M.H., Blagosklonny, M.V., Bender, J., Brooks, R., Piekarz, R.L., Tucker, E., Figg, W.D., Chan, K.K., Goldspiel, B., Fojo, A.T., Balcerzak, S.P., Bates, S.E., 2002. Phase I trial of the histone deacetylase inhibitor, depsipeptide (FR901228, NSC 630176), in patients with refractory neoplasms. *Clin. Cancer Res.* 8, 718–728.
- Shedden, K., Te Vruchte, D., Rudenko, G., 2004. Bloodstream form-specific up-regulation of silent vsg expression sites and procyclin in *Trypanosoma brucei* after inhibition of DNA synthesis or DNA damage. *J. Biol. Chem.* 279, 13363–13374.
- Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J.T., Bokesch, H., Kenney, S., Boyd, M.R., 1990. New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst.* 82, 1107–1112.
- Steele, N.L., Plumb, J.A., Vidal, L., Tjornelund, J., Knoblauch, P., Buhl-Jensen, P., Molife, R., Brown, R., De Bono, J.S., Evans, T.R., 2011. Pharmacokinetic and pharmacodynamic properties of an oral formulation of the histone deacetylase inhibitor Belinostat (PXD101). *Cancer Chemother. Pharmacol.* 67, 1273–1279.
- Stolf, D.A., Marek, M., Lancelot, J., Hauser, A.T., Walter, A., Leproult, E., Melesina, J., Rumpf, T., Wurtz, J.M., Cavarelli, J., Sippl, W., Pierce, R.J., Romier, C., Romier, C., Jung, M., 2014. Molecular basis for the antiparasitic activity of a mercaptoacetamide derivative that inhibits histone deacetylase 8 (HDAC8) from the human pathogen *Schistosoma mansoni*. *J. Mol. Biol.* 426 (20), 3442–3453.
- Strahl, B.D., Allis, C.D., 2000. The language of covalent histone modifications. *Nature* 403, 41–45.
- Sumanadasa, S.D., Goodman, C.D., Lucke, A.J., Skinner-Adams, T., Sahama, I., Haque, A., Do, T.A., Mcfadden, G.I., Fairlie, D.P., Andrews, K.T., 2012. Antimalarial activity of the anticancer histone deacetylase inhibitor SB939. *Antimicrob. Agents Chemother.* 56, 3849–3856.
- Sun, W., Tanaka, T.Q., Magle, C.T., Huang, W., Southall, N., Huang, R., Dehdashti, S.J., Mckew, J.C., Williamson, K.C., Zheng, W., 2014. Chemical signatures and new drug targets for gametocytocidal drug development. *Sci. Rep.* 4, 3743.
- Sundriyal, S., Malmquist, N.A., Caron, J., Blundell, S., Liu, F., Chen, X., Srimongkolpithak, N., Jin, J., Charman, S.A., Scherf, A., Fuchter, M.J., 2014. Development of diaminoquinazoline histone lysine methyltransferase inhibitors as potent blood-stage antimalarial compounds. *ChemMedChem.* 9, 2360–2373.
- Sykes, M.L., Avery, V.M., 2009. Development of an Alamar blue viability assay in 384-well format for high throughput whole cell screening of *Trypanosoma brucei* bloodstream form strain 427. *Am. J. Trop. Med. Hyg.* 81, 665–674.
- Thompson, C.A., 2014. Belinostat approved for use in treating rare lymphoma. *Am. J. Health Syst. Pharm.* 71, 1328.
- Tonkin, C.J., Carret, C.K., Duraisingh, M.T., Voss, T.S., Ralph, S.A., Hommel, M., Duffy, M.F., Silva, L.M., Scherf, A., Ivens, A., Speed, T.P., Beeson, J.G., Cowman, A.F., 2009. Sir2 paralogs cooperate to regulate virulence genes and antigenic variation in *Plasmodium falciparum*. *PLoS Biol.* 7, e84.
- Trenholme, K., Marek, L., Duffy, S., Pradel, G., Fisher, G., Hansen, F.K., Skinner-Adams, T.S., Butterworth, A., Ngwa, C.J., Moeking, J., Goodman, C.D., Mcfadden, G.I., Sumanadasa, S.D., Fairlie, D.P., Avery, V.M., Kurz, T., Andrews, K.T., 2014. Lysine acetylation in sexual stage malaria parasites is a target for antimalarial small molecules. *Antimicrob. Agents Chemother.* 58, 3666–3678.
- Walliker, D., Quakyi, I.A., Wellems, T.E., McCutchan, T.F., Szarfman, A., London, W.T., Corcoran, L.M., Burkot, T.R., Carter, R., 1987. Genetic analysis of the human malaria parasite *Plasmodium falciparum*. *Science* 236, 1661–1666.
- Wang, Q.P., Kawahara, T., Horn, D., 2010. Histone deacetylases play distinct roles in telomeric VSG expression site silencing in African trypanosomes. *Mol. Microbiol.* 77, 1237–1245.
- Wellems, T.E., Oduola, Ayo M.J., Fenton, B., Desjardins, R., Panton, L.J., do Rosario, V.E., 1988. Chromosome size variation occurs in cloned *Plasmodium falciparum* on *in vitro* cultivation. *Rev. Bras. Genet.* 11, 813–825.
- WHO, 2008. Global Burden of Disease: 2004 Update (Geneva).
- Xu, W.S., Parmigiani, R.B., Marks, P.A., 2007. Histone deacetylase inhibitors: molecular mechanisms of action. *Oncogene* 26, 5541–5552.
- Yeo, W., Chung, H.C., Chan, S.L., Wang, L.Z., Lim, R., Picus, J., Boyer, M., Mo, F.K., Koh, J., Rha, S.Y., Hui, E.P., Jeung, H.C., Roh, J.K., Yu, S.C., To, K.F., Tao, Q., Ma, B.B., Chan, A.W., Tong, J.H., Erlichman, C., Chan, A.T., Goh, B.C., 2012. Epigenetic therapy using belinostat for patients with unresectable hepatocellular carcinoma: a multicenter phase I/II study with biomarker and pharmacokinetic analysis of tumors from patients in the Mayo phase II consortium and the cancer therapeutics research group. *J. Clin. Oncol.* 30, 3361–3367.